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## Ethanol potentiates hypoxic liver injury: role of hepatocyte $\text{Na}^+$ overload

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### Abstract

Centrilobular hypoxia has been suggested to contribute to hepatic damage caused by alcohol intoxication. However, the mechanisms involved are still poorly understood. We have investigated whether alterations of  $\text{Na}^+$  homeostasis might account for ethanol-mediated increase in hepatocyte sensitivity to hypoxia. Addition of ethanol (100 mmol/l) to isolated rat hepatocytes incubated under nitrogen atmosphere greatly stimulated cell death. An increase in intracellular  $\text{Na}^+$  levels preceded cell killing and  $\text{Na}^+$  levels in hepatocytes exposed to the combination of ethanol and hypoxia were almost twice those in hypoxic cells without ethanol.  $\text{Na}^+$  increase was also observed in hepatocytes incubated with ethanol in oxygenated buffer. Ethanol addition significantly lowered hepatocyte pH. Inhibiting ethanol and acetaldehyde oxidation with, respectively, 4-methylpyrazole and cyanamide prevented this effect. 4-methylpyrazole, cyanamide as well as hepatocyte incubation in a  $\text{HCO}_3^-$ -free buffer or in the presence of  $\text{Na}^+/\text{H}^+$  exchanger blocker 5-(*N,N*-dimethyl)-amiloride also reduced  $\text{Na}^+$  influx in ethanol-treated hepatocytes. 4-methylpyrazole and cyanamide similarly prevented ethanol-stimulated  $\text{Na}^+$  accumulation and hepatocyte killing during hypoxia. Moreover, ethanol-induced  $\text{Na}^+$  influx caused cytotoxicity in hepatocytes pre-treated with  $\text{Na}^+,\text{K}^+$ -ATPase inhibitor ouabain. Also in this condition 4-methylpyrazole and 5-(*N,N*-dimethyl)-amiloride decreased cell killing. These results indicate that ethanol can promote cytotoxicity in hypoxic hepatocytes by enhancing  $\text{Na}^+$  accumulation. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Sodium; Acidosis; Hypoxia; Cell death; Alcohol related liver injury

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### 1. Introduction

It is well established that in both humans and experimental animals chronic ethanol consumption leads to liver damage in oxygen-poor centrilobular areas of the liver lobule [1]. In the early 1970s Videla and co-workers first observed an increased oxygen consumption by liver slices prepared from rats exposed chronically to ethanol [2]. A two fold increase

in oxygen uptake was also detected in isolated perfused liver following a single large dose of ethanol [3]. Although ethanol stimulates hepatic blood supply by increasing portal blood flow [4,5], such an increased hepatic oxygen delivery cannot compensate ethanol-stimulated oxygen demand. Indeed, an increased oxygen extraction associated with a 40% lowering in the  $\text{pO}_2$  of hepatic venous blood can be detected in the liver of chronic alcohol-fed rats [6].

These observations lead to the hypothesis that the imbalance between an increased hepatic oxygen consumption and a decreased oxygen delivery to the liver can contribute to the development of centrilob-

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ular hypoxia in livers exposed to ethanol [3,7]. Consistently, Arteel and co-workers have demonstrated that both acute and chronic alcohol treatment cause the staining of centrilobular areas by the hypoxia-sensitive dye pimonidazole [8,9].

The possibility that ethanol-mediated alterations in oxygen delivery to centrilobular areas might contribute to alcoholic liver injury has received several experimental supports. Centrilobular necrosis develops in the livers of rats chronically fed with alcohol when oxygen supply is reduced [10]. Furthermore, the addition of ethanol stimulates cytotoxicity in either isolated perfused livers [11] or isolated hepatocytes exposed to hypoxic conditions [12,13]. However, the biochemical mechanisms responsible for the enhancement of hypoxic damage by ethanol have not been characterized.

We and others have reported that in isolated hepatocytes exposed to mitochondrial inhibitors, hypoxia or oxidative stress intracellular  $\text{Na}^+$  accumulation preceded the onset of cytotoxicity [14–16]. In these conditions cytoprotection can be obtained by the substitution of  $\text{NaCl}$  in the incubation medium with equimolar amount of choline chloride [14,15] or by preventing  $\text{Na}^+$  increase with glycine or strychnine that interfered with  $\text{Na}^+$ -associated  $\text{Cl}^-$  influx [17]. Consistently, liver pre-treatment with furesimide and bumetamide, two inhibitors of the  $\text{Na}^+/\text{K}^+$ - $2\text{Cl}^-$  co-transporter also decreased  $\text{Na}^+$  accumulation and tissue injury in isolated perfused livers undergoing warm ischemia [18].

These observations prompted us to investigate whether ethanol might promote cytotoxicity in hypoxic hepatocytes by enhancing  $\text{Na}^+$  accumulation.

## 2. Materials and methods

Collagenase (Type I), *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid) (Hepes), propidium iodide, 4-methylpyrazole, cyanamide and nigericin were purchased from Sigma (St Louis, MO). 5-(*N,N*-Dimethyl)-amiloride and 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein-acetoxyethyl ester (BCECF-AM) were obtained from, respectively, Calbiochem-Novabiochem (San Diego, CA) and Molecular Probes (Eugene, OR). Percoll was supplied by Pharmacia (Uppsala, Sweden). All the other chemi-

cals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Male Wistar rats (180–250 g weight) were obtained from Nossan (Correzzana, Italy) and allowed free access to water and food. Isolated rat hepatocytes were prepared by liver perfusion with collagenase as previously reported [15]. Cell viability, estimated at the beginning of the experiments, ranged between 85% and 90%. Hepatocytes were suspended in Krebs–Henseleit–Hepes (KHH) buffer containing 118 mmol/l  $\text{NaCl}$ , 4.7 mmol/l  $\text{KCl}$ , 1.2 mmol/l  $\text{KH}_2\text{PO}_4$ , 1.3 mmol/l  $\text{CaCl}_2$ , 25 mmol/l  $\text{NaHCO}_3$  and 20 nmol/l Hepes at pH 7.4, and incubated at 37°C (final cell density of  $10^6/\text{ml}$ ) in 50-ml glass bottles under continuous fluxing with 95%  $\text{O}_2$ –5%  $\text{CO}_2$ . For experiments performed under hypoxic conditions KHH buffer was equilibrated with 95%  $\text{N}_2$ –5%  $\text{CO}_2$  and hepatocytes were incubated in sealed bottles under 95%  $\text{N}_2$ –5%  $\text{CO}_2$  atmosphere. For the experiments performed in the absence of  $\text{Na}^+$ ,  $\text{NaCl}$  and  $\text{NaHCO}_3$  were replaced by 118 mmol/l choline chloride and 25 mmol/l  $\text{KHCO}_3$ , respectively. The bicarbonate-free KHH buffer contained 143 mmol/l  $\text{NaCl}$  and was fluxed with 95%  $\text{O}_2$ –5%  $\text{N}_2$  gas mixture. Hepatocyte treatment with the different inhibitors was performed by 10 min preincubation at 37°C before ethanol addition.

Cell viability was estimated by microscope-counting the hepatocyte excluding Trypan blue and by the determination of nuclear fluorescence staining with propidium iodide according to the method described by Gores et al. [19]. For this latter assay, 1 ml aliquots of the cell suspensions were taken, diluted 10 times in KHH buffer containing 20  $\mu\text{g}/\text{ml}$  propidium iodide, and the fluorescence intensity was immediately measured at 515/620 nm wavelength pair. The values were expressed as percent of the total propidium iodide fluorescence measured in each sample after cell permeabilization with digitonin (375  $\mu\text{mol}/\text{l}$ ).

Hepatocyte ATP content was estimated by reverse-phase HPLC, after protein precipitation with perchloric acid, using a Hibar Lichrospher 100RP-18 column (Merck) and 0.1 M potassium phosphate buffer (pH 5.3) as eluent according to Debetto and Bianchi [20].

Cytosolic pH was measured using the fluorescent indicator 2',7'-bis-(carboxyethyl)-5,6-carboxyfluores-

cein-acetoxymethyl ester (BCECF-AM) as described by Borle et al. [21]. Isolated hepatocytes ( $10^6$  cells/ml) were loaded with BCECF-AM (5 µg/ml) by 15 min incubation at 37°C in a modified KHH buffer containing 2% bovine serum albumin. After washing, the cells were re-suspended in fresh oxygenated KHH medium and incubated 20 min at 37°C with ethanol (100 mmol/l) with or without 4-methylpyrazole or cyanamide. At the end of the incubation, 2-ml aliquots were taken and centrifuged for 1 min at 1000 rpm. Cell pellets were re-suspended in fresh medium and the fluorescence was determined at 450/530 nm wavelength pair using a Hitachi 4500 spectrofluorimeter. Calibration values were obtained for each experiment by incubating hepatocytes in media at different pH containing 10 µmol/l  $K^+/H^+$  ionophore nigericin and 120 mmol/l  $K^+$ .

Intracellular  $Na^+$  levels were measured in viable hepatocytes separated by centrifugation through 3 ml of Percoll solution ( $d=1.06$ ) in 0.25 mol/l sucrose, as previously reported [15]. After centrifugation, the Percoll solution was rapidly removed by aspiration, and the cell pellets were extracted with 0.5 ml of 0.8 N perchloric acid.  $Na^+$  was measured in aliquots of the protein-free acidic supernatants diluted 200 times with distilled water using a Varian

AA-1475 atomic absorption spectrophotometer (Varian Instruments, Palo Alto, CA). The values were corrected for the protein content of each pellet. Intracellular  $Na^+$  concentrations measured by this method were comparable with the determination of cytosolic  $Na^+$  levels using the fluorescent  $Na^+$  probe benzofuran isophthalate (SBFI) [22].

Statistical analysis for multiple comparisons was performed by one-way analysis of variance with Bonferroni's corrections. Distribution normality of the groups considered was preliminarily evaluated by the Shapiro-Wilk test.

### 3. Results

The incubation of freshly isolated rat hepatocytes in the presence of 100 mmol/l ethanol did not appreciably affected ATP content and cell viability (Fig. 1). Cytotoxicity was instead evident in hepatocytes incubated under hypoxic conditions (Fig. 1). However, the concomitant presence of ethanol greatly increased cell killing caused by hypoxia (Fig. 1). This effect was not related to the impairment of energy production, since ethanol did not further worsen ATP depletion in hypoxic hepatocytes (Fig. 1). The

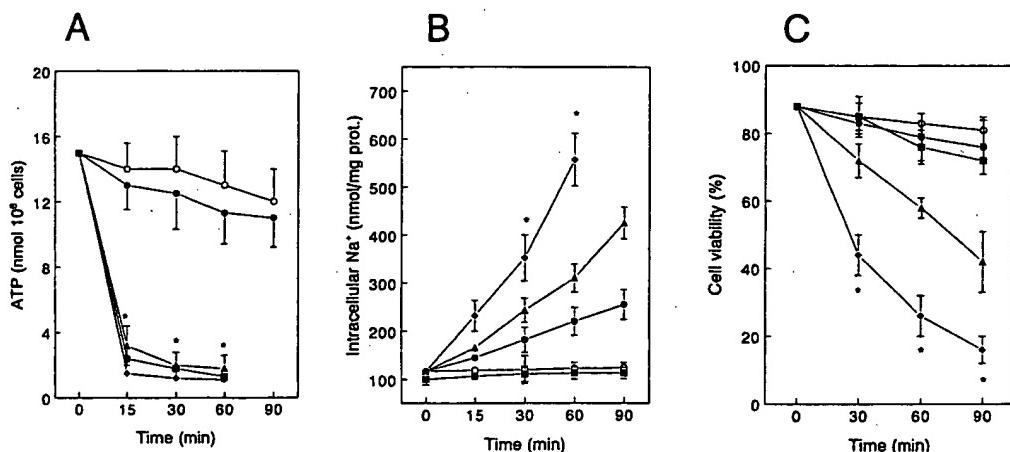


Fig. 1. Effects of ethanol addition on intracellular ATP levels (A),  $Na^+$  content (B) and cell viability (C) of isolated rat hepatocytes incubated under normoxic or hypoxic conditions. Isolated hepatocytes ( $10^6$  cells/ml) were incubated at 37°C with or without ethanol (100 mmol/l) in open bottles under continuous fluxing with 95%  $O_2$ -5%  $CO_2$  or in sealed bottles under 95%  $N_2$ -5%  $CO_2$  atmosphere. Key: control cells incubated under normoxic (○) or hypoxic (▲) conditions; hepatocytes exposed to ethanol under normoxic (●) or hypoxic (◆) conditions; hepatocytes exposed to both ethanol and hypoxia in a  $Na^+$ -free KHH buffer (■). The results are means of 5–7 different experiments  $\pm$  S.D. Statistical significance: \* $P < 0.01$  versus hypoxic cells without ethanol or normoxic hepatocytes receiving ethanol.

measurement of intracellular  $\text{Na}^+$  content demonstrated that the addition of ethanol greatly stimulated  $\text{Na}^+$  accumulation induced by hypoxia (Fig. 1).  $\text{Na}^+$  overload was blocked by cell incubation in a  $\text{Na}^+$ -free medium, that also completely prevented cytotoxicity in hypoxic ethanol-treated hepatocytes (Fig. 1). Fig. 1 shows that a twofold increase of  $\text{Na}^+$  levels was also appreciable in hepatocytes incubated with ethanol under normoxic conditions. Further experiments using hepatocytes exposed to ethanol (100 mmol/l) in oxygenated buffer demonstrated that inhibiting ethanol and acetaldehyde metabolism by, respectively, 4-methylpyrazole (0.5 mmol/l) or cyanamide (10  $\mu\text{mol/l}$ ) blocked ethanol-mediated increase in intracellular  $\text{Na}^+$  (Fig. 2). A similar effect was also obtained by the addition of  $\text{Na}^+/\text{H}^+$  exchanger inhibitor 5-(*N,N*'-dimethyl)-amiloride (DMA) (10  $\mu\text{mol/l}$ ) or by hepatocyte incubation in a  $\text{HCO}_3^-$ -free buffer (Fig. 2). This suggested the possibility that the activation of cellular acid buffering systems might trigger  $\text{Na}^+$  influx. Measurement of

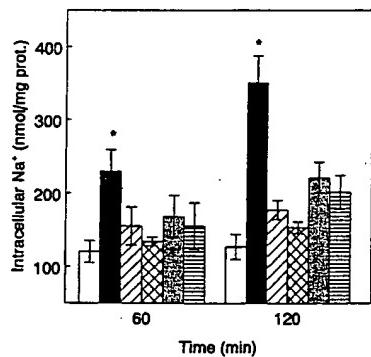


Fig. 2. Inhibitors of ethanol and acetaldehyde metabolism or treatments interfering with the activity of cellular acid buffering systems reduce intracellular  $\text{Na}^+$  accumulation in isolated hepatocytes incubated with ethanol under normoxic conditions. Isolated hepatocytes ( $10^6$  cells/ml) were incubated at 37°C with or without ethanol (100 mmol/l) in open bottles under continuous fluxing with 95%  $\text{O}_2$ –5%  $\text{CO}_2$ . The bars represent: control cells without ethanol (open bars); hepatocytes exposed to ethanol (filled bars); hepatocytes receiving ethanol plus 4-methylpyrazole (0.5 mmol/l) (hatched bars), plus cyanamide (10  $\mu\text{mol/l}$ ) (double hatched bars) or plus 5-(*N,N*-dimethyl)-amiloride (10  $\mu\text{mol/l}$ ) (dotted bars); hepatocytes incubated with ethanol in a  $\text{HCO}_3^-$ -free buffer (stripped bars). The results are means of 3–4 different experiments  $\pm$  S.D. Statistical significance: \* $P < 0.01$  versus controls and versus cells incubated with ethanol plus the different inhibitors.

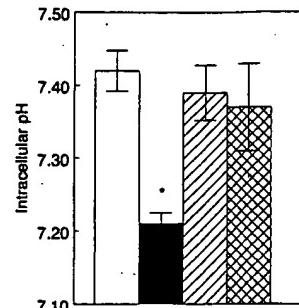


Fig. 3. Effect of ethanol metabolism on hepatocyte cytosolic pH. Isolated hepatocytes ( $10^6$  cells/ml) were loaded with BCECF-AM (5  $\mu\text{g/ml}$ ) by 15 min incubation at 37°C in a modified KHH buffer containing 2% bovine serum albumin. After washing, the cells were re-suspended in fresh KHH medium and incubated 20 min at 37°C in oxygenated KHH buffer with or without ethanol (100 mmol/l) and the different inhibitors. The bars represent: control hepatocytes without ethanol (open bar); hepatocytes treated with ethanol (filled bar); hepatocytes treated with ethanol in the presence of 4-methylpyrazole (0.5 mmol/l) (hatched bar) or cyanamide (10  $\mu\text{mol/l}$ ) (double hatched bar). The results are means of four different experiments  $\pm$  S.D. Statistical significance: \* $P < 0.05$  versus controls.

intracellular pH with the fluorescent dye BCECF-AM confirmed that ethanol addition significantly lowered hepatocyte pH from  $7.42 \pm 0.03$  to  $7.21 \pm 0.09$  ( $P < 0.05$ ) (Fig. 3). Hepatocyte pre-treatment with 4-methylpyrazole and cyanamide inhibited ethanol-induced pH decrease (Fig. 3). However, these inhibitors did not interfere with cellular pH in hepatocytes not receiving ethanol (not shown). Interestingly, the pre-treatment with 4-methylpyrazole or cyanamide prevented alcohol-induced stimulation of  $\text{Na}^+$  accumulation and reduced cell killing in hypoxic ethanol-treated hepatocytes (Fig. 4). On the other hand,  $\text{Na}^+$  increase and cytotoxicity were evident when hepatocytes were incubated under hypoxic conditions in the presence of 25 mmol/l sodium acetate, the final product of ethanol oxidation (Fig. 4). This suggested that the lowering of intracellular pH resulting from the accumulation of ethanol oxidation products might play an important role in promoting  $\text{Na}^+$  overload and cytotoxicity in ethanol-treated hypoxic hepatocytes. To further verify this possibility hepatocytes were incubated with ethanol under normoxic conditions and the effect of ATP depletion on  $\text{Na}^+$  extrusion was mimicked by inhibiting plasma membrane  $\text{Na}^+,\text{K}^+$ -ATPase with ouabain. Fig. 5, demonstrates that blocking  $\text{Na}^+$  extrusion with oua-

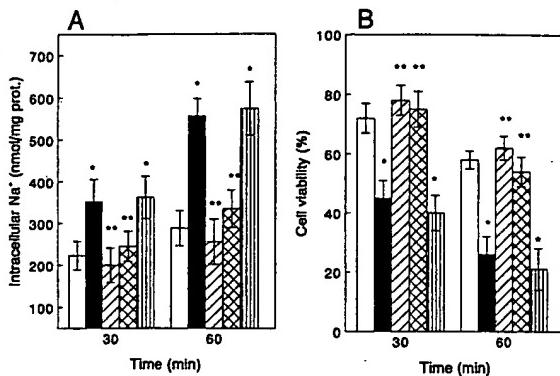


Fig. 4.  $\text{Na}^+$  overload and hepatocyte killing by hypoxia in hepatocytes receiving ethanol or acetate and effect of inhibiting ethanol and acetaldehyde oxidation with, respectively, 4-methylpyrazole and cyanamide. Isolated hepatocytes ( $10^6$  cells/ml) were incubated at  $37^\circ\text{C}$  with or without ethanol (100 mmol/l) in sealed bottles under 95%  $\text{N}_2$ -5%  $\text{CO}_2$  atmosphere.  $\text{Na}^+$  content (A) and cell viability (B) were measured as described in Section 2. The bars represent: control cells incubated under hypoxic conditions (open bars); hepatocytes exposed to ethanol and hypoxia (filled bars); hepatocytes exposed to ethanol and hypoxia in the presence of 4-methylpyrazole (0.5 mmol/l) (hatched bars) or cyanamide (10  $\mu\text{mol/l}$ ) (double hatched bars); hypoxic hepatocytes receiving sodium acetate (25 mmol/l) instead of ethanol (stripped bars). The results are means of 3–5 different experiments  $\pm$  S.D. Statistical significance: \* $P < 0.05$  versus cells incubated under hypoxia without additions; \*\* $P < 0.05$  versus ethanol-treated hypoxic hepatocytes.

bain (1 mmol/l) did not appreciably modify  $\text{Na}^+$  content in control hepatocytes, but further stimulated  $\text{Na}^+$  accumulation induced by the addition of 100 mmol/l ethanol. Cytotoxicity developed in hepatocytes exposed to both ethanol and ouabain (Fig. 5). However, blocking ethanol oxidation with 4-methylpyrazole or  $\text{Na}^+/\text{H}^+$  exchanger with DMA completely protected against  $\text{Na}^+$  accumulation and cytotoxicity induced by the combination of ethanol and ouabain (Fig. 5).

#### 4. Discussion

It is well established that alcohol stimulates oxygen uptake by the liver, a condition, also known as liver hypermetabolic state [3,4]. Although ethanol enhances hepatic blood supply by increasing portal blood flow [6,7], this is not sufficient to compensate the increased oxygen extraction and alcohol-treated liv-

ers show a 40% lowering of hepatic vein  $\text{pO}_2$  [7]. Beside causing centrilobular hypoxia, chronic ethanol consumption also affects liver ATP production under hypoxic conditions [23]. Ivester and co-workers have observed that, upon incubation under very low oxygen tensions, ATP concentration and energy charge in hepatocytes isolated from alcohol-fed rats are significantly lower than in cell preparations from control animals [12]. Such a difference has been ascribed to a reduced efficiency of ATP synthesis in the mitochondria as well as to a depression of anaerobic glycolysis due to the depletion of glycogen content [23,24]. These events can explain the increased susceptibility to hypoxic damage of hepatocytes isolated from chronic alcohol-fed rats as compared to liver cells obtained from pair-fed controls [12,25]. However, other mechanisms should also be involved, since the addition of ethanol *in vitro* potentiates cell killing by hypoxia also in hepatocyte suspensions from untreated rats [13]. Similarly, acute ethanol administration greatly stimulates liver injury in isolated liver perfused under hypoxic conditions [11].

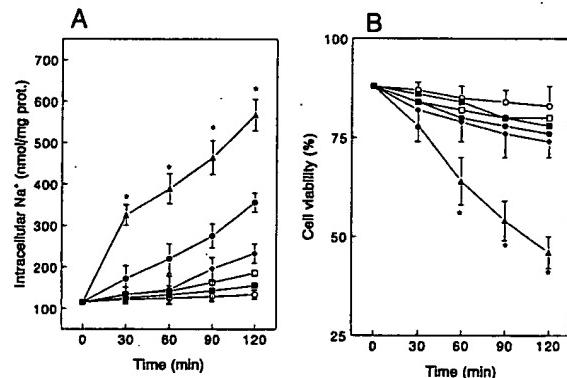


Fig. 5. Role of  $\text{Na}^+$  overload and in the development of ethanol hepatotoxicity following inhibition of plasma membrane  $\text{Na}^+/\text{K}^+$ -ATPase by ouabain. Isolated hepatocytes ( $10^6$  cells/ml) were incubated at  $37^\circ\text{C}$  with or without ethanol (100 mmol/l) or ouabain (1 mmol/l) in open bottles under continuous fluxing with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ .  $\text{Na}^+$  content (A) and cell viability (B) were measured as described in Section 2. Key: control cells without additions (○); hepatocytes receiving ouabain (□) or ethanol (●) alone; hepatocytes incubated with the combination of ethanol and ouabain (▲); hepatocytes receiving ethanol and ouabain along with 4-methyl-pyrazole (0.5 mmol/l) (■) or 5-(*N,N*-dimethyl)-amiloride (10  $\mu\text{mol/l}$ ) (◆). The results are means of three different experiments  $\pm$  S.D. Statistical significance: \* $P < 0.01$  versus cells incubated with ethanol alone or ethanol plus ouabain and either 4-methylpyrazole or DMA.

The results of the present study show that the addition of ethanol increases intracellular  $\text{Na}^+$  content in isolated hepatocytes incubated in the presence of oxygen and enhances by almost twofold  $\text{Na}^+$  accumulation occurring during hypoxic treatment.  $\text{Na}^+$  overload occurring during hypoxia or metabolic inhibition mainly results from the combined block of ATP-dependent  $\text{Na}^+$  efflux through the  $\text{Na}^+,\text{K}^+$ -ATPase and from the stimulation of  $\text{Na}^+$  influx in response to cytosolic acidification [15]. Indeed, the lowering of cytosolic pH is a powerful stimulus for the activation of  $\text{Na}^+$ -mediated acid buffering systems involving  $\text{Na}^+/\text{H}^+$  exchanger and of  $\text{Na}^+/\text{HCO}_3^-$  co-transporter [26]. In vitro ethanol addition does not interfere with hepatocyte energy production. However, an appreciable lowering in cytosolic pH can be observed in hepatocytes receiving ethanol while incubated in the presence of oxygen. This effect is prevented by blocking ethanol and acetaldehyde metabolism with, respectively, 4-methylpyrazole and cyanamide, indicating that the formation of acetate and lactate as end-products of ethanol oxidation might be responsible for hepatocellular acidification. Indeed, an hyperproduction of lactic acid, leading to hyperlactacidemia, is among the metabolic alterations caused by the excess NADH occurring during liver ethanol oxidation [27]. Nonetheless, Benedetti and colleagues have proposed that interference by ethanol with the activity of  $\text{H}^+$  transport systems might also contribute to the lowering of intracellular pH in hepatocytes isolated from alcohol-fed rats [28].

The link between ethanol metabolism, intracellular acidosis and alterations of  $\text{Na}^+$  homeostasis is supported by the observation that the addition of acetate, the final product of ethanol oxidation, also promote  $\text{Na}^+$  overload in hypoxic hepatocytes. Conversely, inhibiting ethanol conversion to acetate by 4-methylpyrazole or cyanamide as well as blocking the acid buffering systems prevents  $\text{Na}^+$  increase. It is noteworthy that 4-methylpyrazole and 5-(*N,N'*-dimethyl)-amiloride prevent ethanol-induced  $\text{Na}^+$  accumulation and cytotoxicity in oxygenated hepatocyte suspensions pre-treated with ouabain, in order to mimic the block of  $\text{Na}^+$  extrusion through  $\text{Na}^+,\text{K}^+$ -ATPase due to ATP depletion by hypoxia. However, it cannot be excluded that other mechanisms might also contribute to cause  $\text{Na}^+$  overload. Von Dalh and Häussinger have reported that acetal-

dehyde might activate  $\text{Na}^+,\text{K}^+,\text{Cl}^-$  co-transporter in perfused livers exposed to ethanol [29]. Furthermore, ethanol is known to promote oxidative stress by stimulating the formation of reactive oxygen species (ROS) within the hepatocytes [30]. Recently, Khan and O'Brien have reported that an increased ROS production is associated with ethanol-mediated potentiation of hepatocyte killing by hypoxia [13]. In such a condition oxidative damage might further contribute to  $\text{Na}^+$  accumulation by impairing the activity of plasma membrane  $\text{Na}^+,\text{K}^+$ -ATPase.

Alterations in hepatocyte volume regulations play an important role in the development of irreversible cell injury consequent to  $\text{Na}^+$  overload induced by hypoxia or metabolic inhibition [14,31]. A moderate hepatic swelling (about 9% of the initial volume) has also been observed following the infusion of ethanol (1–100 mmol/l) into aerobically perfused livers [29]. This effect can be prevented by the addition of 4-methylpyrazole as well as by inhibitors of  $\text{Na}^+$  transport [29], indicating that ethanol interference with hepatic ion distribution can worsen osmotic imbalances consequent to hypoxia.

In conclusion, we propose that alterations of  $\text{Na}^+$  homeostasis can represent one of the mechanisms by which ethanol can aggravate hypoxic liver injury.

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